Biophysical Journal Volume 76 May 1999 2575–2586

Binding of Peripheral Proteins to Mixed Lipid Membranes: Effect of Lipid Demixing upon Binding

Thomas Heimburg, Brigitta Angerstein, and Derek Marsh

Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, D-37070 Göttingen, Germany

ABSTRACT Binding isotherms have been determined for the association of horse heart cytochrome *c* with dioleoyl phosphatidylglycerol (DOPG)/dioleoyl phosphatidylcholine (DOPC) bilayer membranes over a range of lipid compositions and ionic strengths. In the absence of protein, the DOPG and DOPC lipids mix nearly ideally. The binding isotherms have been analyzed using double layer theory to account for the electrostatics, either the Van der Waals or scaled particle theory equation of state to describe the protein surface distribution, and a statistical thermodynamic formulation consistent with the mass-action law to describe the lipid distribution. Basic parameters governing the electrostatics and intrinsic binding are established from the binding to membranes composed of anionic lipid (DOPG) alone. Both the Van der Waals and scaled particle equations of state can describe the effects of protein distribution on the DOPG binding isotherms equally well, but with different values of the maximum binding stoichiometry (13 lipids/protein for Van der Waals and 8 lipids/protein for scaled particle theory). With these parameters set, it is then possible to derive the association constant, K_r, of DOPG relative to DOPC for surface association with bound cytochrome *c* by using the binding isotherms obtained with the mixed lipid membranes. A value of K_r (DOPG:DOPC) = 3.3–4.8, depending on the lipid stoichiometry, is determined that consistently describes the binding at different lipid compositions and different ionic strengths. Using the value of K_r obtained it is possible to derive the average in-plane lipid distribution and the enhancement in protein binding induced by lipid redistribution using the statistical thermodynamic theory.

INTRODUCTION

Basic peripheral proteins are bound to membranes largely by electrostatic forces mediated by the negatively charged lipid component in the membrane (Sankaram and Marsh, 1993). Of considerable interest with respect to the membrane is the extent to which negatively charged lipids are recruited to the vicinity of the protein (Fig. 1, *middle*), which potentially could give rise to formation of in-plane membrane domains. Additionally, a redistribution of the lipids will enhance the apparent binding affinity of the protein relative to that for a random lipid distribution in membranes of heterogeneous lipid composition. The maximum degree of protein binding is expected for a complete demixing of charged and zwitterionic lipid components in the membrane, possibly in pre-existing domains (Fig. 1, *bottom*). On the other hand, dissociation of lipid domains, or any process that causes a transition toward a more homogeneous lipid mixture (Fig. 1, *top*), will decrease the extent of protein binding and hence affords a means for controlling the membrane-protein association. The latter is of functional significance for, e.g., the activation of protein kinase C (Mosior and Newton, 1995). The three limiting cases of different lipid distributions are illustrated schematically in Fig. 1.

Received for publication 2 February 1998 and in final form 27 January 1999.

Address reprint requests to Dr. Thomas Heimburg, Max-Planck-Institut für biophysikalische Chemie, D-37070 Göttingen, Germany. Tel.: 49-551-201-1412; Fax: 49-551-201-1501; E-mail: theimbu@gwdg.de; Website: http://www.gwdg.de/~theimbu.

0006-3495/99/05/2575/12 $2.00

Domain formation on binding basic proteins or peptides has been observed both in natural membranes and in lipid membranes containing charged and zwitterionic species using fluorescence microscopy (Rodgers and Glaser, 1991; Haverstick and Glaser, 1989; Yang and Glaser, 1995). The large size of the domains relative to the negatively charged lipid content in these cases suggests, however, that they are stabilized by long-range effects rather than by a direct local selectivity for a particular lipid type (Kleinschmidt and Marsh, 1997; Ben Tal et al., 1996). Investigation of the assembly of the latter type of domains and of small, localized domains in general still poses a considerable challenge.

Selectivity for the interaction of negatively charged lipids with peripheral basic proteins and peptides has been observed by spectroscopic techniques in mixtures with zwitterionic lipids (Sankaram and Marsh, 1993). For example, selectivity series have been established for the perturbation of different spin-labeled lipid species at probe amounts in negatively charged lipid membranes to which peripheral proteins are bound (Sankaram et al., 1989a,b; 1990). Preferential interaction of cytochrome *c* with cardiolipin has been found by solid-state ^{31}P-NMR of mixtures with zwitterionic lipids (Pinheiro and Watts, 1994). Somewhat similarly, a preferential interaction of cardiotoxin II with phosphatidylglycerol relative to phosphatidylcholine was found (Carbone and Macdonald, 1996). In this latter case, resolution of the two lipid components was sufficient to permit the construction of relative binding curves for the two lipids from the NMR data. Using ^{2}H-NMR, a preferential interaction of the peptide pentalysine with phosphatidylserine has been observed relative to phosphatidylcholine (Roux et al., 1988). Fluorescence energy transfer measurements have

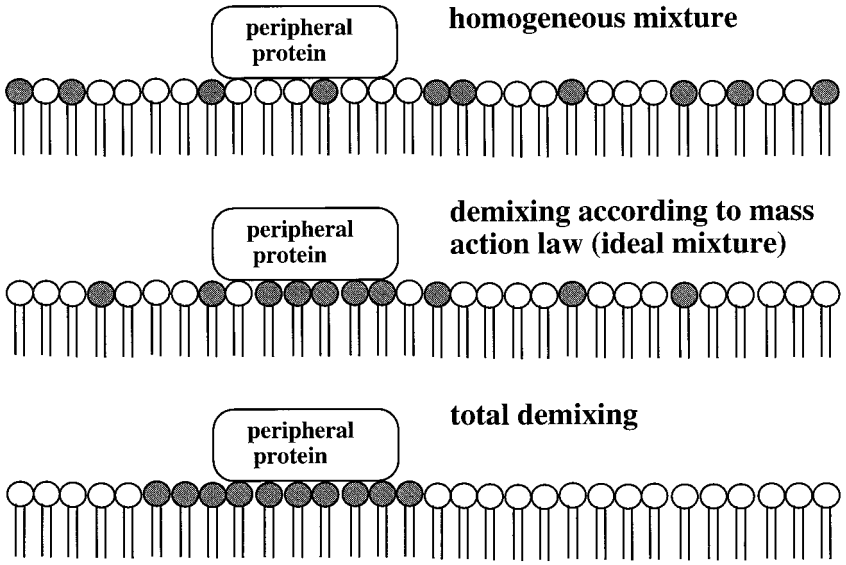

FIGURE 1 Schematic representation of the lipid distribution for different situations of protein binding to mixed membranes composed of charged (*shaded*) and zwitterionic (*open*) lipids. *Top:* a homogeneous lipid mixture in which no lipid rearrangement takes place on binding of the peripheral protein. *Middle:* lipids which mix well and redistribute according to their different affinities for the protein. *Bottom:* immiscible lipids which form in-plane membrane domains in the absence of protein binding.

also indicated a demixing of phosphatidylglycerol and phosphatidylcholine on binding a basic gp40-derived peptide to mixed lipid membranes (Gawrisch et al., 1995).

Although these spectroscopic measurements provide direct evidence for a selectivity in interaction of different lipid species with peripheral proteins, most do not themselves yield quantitative information on the lipid distribution. Nor, with one notable exception (Carbone and Macdonald, 1996), are they capable of yielding estimates for the relative association constants of the different lipids without making certain ad hoc assumptions. To determine the surface association constants, it is necessary to perform experiments that are directly related to the thermodynamics of the interaction. One such way, used here, is to determine the protein binding isotherms with mixed lipid systems.

In the present work we have focused on the binding of cytochrome *c* to mixed lipid membranes composed of dioleoyl phosphatidylglycerol (DOPG) and dioleoyl phosphatidylcholine (DOPC). Phosphatidylglycerols and phosphatidylcholines of identical chain compositions are known to mix almost ideally at neutral pH in the absence of proteins or divalent metal ions (Findlay and Barton, 1978; Garidel et al., 1997). Therefore, their in-plane distribution should respond optimally to the binding of peripheral proteins, according to their intrinsic relative affinities for association with the protein.

Previously, we have been able to describe the ionic strength-dependence of cytochrome *c* binding to membranes composed wholly of a single negatively charged lipid-species by using electrostatic double layer theory, when allowance is made for the distributional free energy of the surface-bound protein using the Van der Waals equation of state (Heimburg and Marsh, 1995). Here, we show that this is equally possible if scaled particle theory for hard discs is used to describe the protein distribution (cf. Chatelier and Minton, 1996). The effective lipid/protein stoichiometry at maximum binding is, however, different in these two cases. On this basis, it is then possible to predict the extent of protein binding to mixed lipid membranes for a given surface distribution of the negatively charged and zwitterionic components. The latter is determined by deriving a statistical thermodynamic expression for the distributional free energy that is characterized by the relative association constant for the two lipids. Somewhat different approaches to this latter problem have been used previously (Cutsforth et al., 1989; Mosior and McLaughlin, 1992a; Carbone and Macdonald, 1996). The advantage of the present method is that it leads directly to analytical expressions for the mean lipid distribution over the complete range of protein binding occupancies.

In this way, it has been possible to give a consistent interpretation of all of the cytochrome *c* binding isotherms obtained at different ionic strengths and different lipid compositions, with the same value for the surface association constant of DOPG relative to DOPC.

MATERIALS AND METHODS

Cytochrome *c* (type VI, oxidized form, Sigma Chemical Co., St. Louis, MO) was used without further purification. DOPG and DOPC (Avanti Polar Lipids, Birmingham, AL) were shown to be pure on thin layer chromatography and were used without further purification.

Lipids were mixed in a dichloromethane/methanol mixture and dried under nitrogen and in a vacuum desiccator. Lipid dispersions (10 mg/ml) and protein solutions (20 mg/ml) were prepared in distilled water. Various amounts of protein solution were added to 0.1 or 1 mg of lipid, respectively, under conditions of minimal ionic strength (i.e., of maximum binding strength). The lipid-protein mixtures were then diluted to a total volume of 6.0 ml (corresponding to 210 μM lipid) with 2 mM Hepes, 1 mM EDTA buffer at pH 7.5, and various concentrations of NaCl in the range of 40–100 mM. The NaCl concentration was adjusted after mixing cytochrome *c* and DOPG in the absence of salt in order to avoid any changes in the accessibility of the protein to the lipid (e.g., multilayer formation) that might occur at higher salt concentrations (Heimburg and Marsh, 1995). The lipid-protein mixtures were then equilibrated at room temperature for 48 h to allow for redistribution of protein from the membrane surface into the buffer. To check on accessibility of the protein

to lipid in mixtures containing DOPC, the hydrated lipid-protein mixtures were subjected to sonication in a Branson bath sonicator. This produced no change in the degree of protein binding. Dispersing the lipid in protein-containing buffer did not change to the extent of binding to DOPG alone, but this preparation protocol was unsuitable for DOPC-containing samples because free protein was trapped, presumably in vesicular structures. The ionic strength was calculated including the counterions of the Hepes and the EDTA in the buffer, a contribution corresponding to 4 mM Na^+. All preparations were under either argon or nitrogen in order to avoid oxidation of the unsaturated lipid chains.

The lipid-protein complexes were separated from the protein free in solution by centrifugation (Beckman L7-55, Ti-50 rotor, 50000 rpm, Beckman Instruments, Fullerton, CA.) for 1 h for samples of high ionic strength and 2 h for samples of low ionic strength. No lipid phosphate was detectable in the supernatant after ultracentrifugation, demonstrating complete resolution of the lipid-protein complex. The concentration of free protein was determined from the spectrophotometric extinction of cytochrome *c* at 546 nm and 410 nm in the supernatant. All protein other than that in the supernatant was assumed to be bound to the lipid membranes.

THEORY

A general statistical thermodynamic expression for the binding isotherm is (Heimburg and Marsh, 1995):

$$\langle i\rangle = [L]K_0 \exp\left(-\frac{d}{di}\left[\frac{\Delta F(i)}{kT}\right]\right) \tag{1}$$

where $\langle i\rangle$ is the mean number of ligands bound to the surface, $[L]$ is the free ligand concentration, K_0 is an intrinsic binding constant, and $\Delta F(i)$ is the overall free energy change on binding i ligands. The binding isotherm is completely determined if the dependence of the change in free energy, $\Delta F(i)$, on the surface occupancy, i, is known. For binding to homogeneous charged lipid membranes, the total free energy change is given by $\Delta F(i) = \Delta F_{el}(i) + \Delta F_D(i)$, where $\Delta F_{el}(i)$ is the electrostatic contribution and $\Delta F_D(i)$ is a distributional term that depends on the surface configuration of the ligands and the lateral interactions between them, including steric repulsion and nonelectrostatic interactions. In the case of binding to inhomogeneous surfaces (for example, a mixed lipid membrane with lateral inhomogeneities in the lipid distribution), the additional term $\Delta F_{LD}(i)$ is included that reflects the change in lipid distribution on ligand binding (see later).

The protein distributional free energy, $\Delta F_D(i)$, is given by the work done to compress the proteins to their local equilibrium surface density. Describing the protein distribution as a two-dimensional van der Waals (VdW) gas on the surface, the distributional free energy is given by (Heimburg and Marsh, 1995):

$$\Delta F_D^{VdW}(i) = -ikT\ln(n-i) - akT\left(\frac{i^2}{n}\right) \tag{2}$$

where n is the maximum number of ligands that can be bound to the surface in a single layer and a is an empirical parameter that describes the interaction between ligands on the surface. Attractive interactions correspond to $a > 0$ and repulsive interactions to $a < 0$. Alternatively, describing the protein distribution by scaled particle theory (SPT) (Reiss et al., 1959) leads to the following expression for the distributional free energy, derived from the SPT equation of state for hard circular discs (Helfand et al., 1961):

$$\Delta F_D^{SPT}(i) = -ikT[\ln(n-i)+1] + \frac{inkT}{n-i} \tag{3}$$

The van der Waals gas model gives a first-order approximation for the distributional free energy. Compared with SPT, which is known to overestimate the hard-disc pressure somewhat (Boublík, 1975), the Van der Waals approximation underestimates the repulsive free energy term, especially in the case of asymmetric ligands (Chatelier and Minton, 1996). The data obtained here for cytochrome *c*, which is an approximately symmetrical ligand, are analyzed equivalently in terms of both formulations.

Binding to a homogeneous charged surface

A mixed lipid membrane with a uniform distribution of the charged component that remains unchanged on binding of the protein ligand (see upper part of Fig. 1) is considered first. The electrostatic contribution to the free energy may be expressed in terms of Gouy-Chapman double layer theory. The electrostatic free energy of a charged surface in an electrolyte is then given by (Jähnig, 1976):

$$F_{el}^{S}(i) = -2q\left(\frac{kT}{e}\right)\ln\left(\frac{-\Lambda_0\sigma}{\sqrt{c}}\right) \tag{4}$$

where q is the overall charge on the surface, σ is the charge density, c is the ionic strength, and Λ_0 is a constant (see Heimburg and Marsh, 1995).

A basic protein with effective charge $+Ze$ is assumed to bind to a homogeneously mixed lipid membrane consisting of negatively charged and uncharged lipid species A and B, respectively. The membrane consists of $n\alpha$ lipids with fraction f_A of the negatively charged component, where α is the number of lipids covered by a single protein. If i proteins are bound to the surface which bears a total lipid charge of $-n\alpha f_A e$, the net charge and charge density of the membrane are, respectively:

$$q = -(n\alpha f_A - iZ)e$$

and

$$\sigma = -\left(f_A - \frac{iZ}{n\alpha}\right)\frac{e}{a_0} \tag{5}$$

where a_0 is the surface area per lipid. From Eqs. 1–5, the binding isotherm for a uniformly charged mixed lipid membrane in the Van der Waals approximation (Eq. 2) is given by (cf. Heimburg and Marsh, 1995):

$$[L] = \frac{1}{K(0,f_A)}\cdot\left(1-\frac{\theta\cdot Z}{f_A\cdot\alpha}\right)^{-2Z}\cdot\frac{\theta}{1-\theta}\cdot\exp\left(\frac{\theta}{1-\theta}-2a\theta\right) \tag{6}$$

whereas the corresponding isotherm obtained by using SPT (i.e., Eq. 3) for the ligand distribution is (cf. Chatelier and Minton, 1996):

$$[L] = \frac{1}{K(0, f_A)} \cdot \left(1 - \frac{\theta \cdot Z}{f_A \cdot \alpha}\right)^{-2Z} \cdot \frac{\theta}{1-\theta} \cdot \exp\left(\frac{3\theta}{1-\theta} + \left(\frac{\theta}{1-\theta}\right)^2\right) \quad (7)$$

where $\theta = i/n$ is the degree of surface coverage by the protein ligands. $K(0, f_A)$ is an intrinsic binding constant that depends on the effective charge of the ligand Z and the fraction of charged lipid f_A:

$$K(0, f_A) = K_0 e^{2Z + \Lambda_2 Z^2} \left(\frac{\Lambda_1}{\sqrt{c}}\right)^{2Z} \left(\frac{\Lambda_3}{\sqrt{c}}\right)^{\Lambda_2 (Z^2/2)} \cdot f_A^{2Z} \equiv K(0) \cdot f_A^{2Z} \quad (8)$$

where the dependence on the ionic strength, c, is given explicitly. $K(0)$ is the intrinsic binding constant for a membrane consisting solely of charged lipids, and Λ_1, Λ_2, and Λ_3 are constants that depend on the size of the ligand, the lipid cross-sectional area, and the temperature (see Heimburg and Marsh, 1995).

These expressions for binding to a uniformly charged mixed lipid membrane without lipid redistribution are very similar to those for a membrane consisting solely of charged lipids. They differ from the latter only in that the charge density of the lipid surface is reduced by a constant factor f_A.

Binding to a surface with complete lipid demixing

The simplest case of a heterogeneously charged surface corresponds to total demixing of the charged and uncharged lipid components into macroscopic domains (Fig. 1, *bottom*). In this case, binding takes place only to the charged lipid domains with a total size of $f_A n\alpha$ lipids, where f_A is the fraction of charged lipid. Binding to these regions is of equal strength to the binding to membranes consisting wholly of charged lipids (characterized by $f_A = 1$ in the above equations). The binding isotherms for membranes with complete lipid demixing are therefore given by Eqs. 6–8, in which $\theta = i/n$ is replaced by $i/(n \cdot f_A)$ and f_A is replaced by $f_A = 1$ at each occurrence. Here it is assumed that the size of the macroscopic domains of the charged lipid is much larger than the Debye length that characterizes screening of long-range electrostatic interactions by the electrolyte.

Lipid redistribution upon ligand binding

In general, the negatively charged lipids will redistribute in the plane of the membrane in response to protein binding (Fig. 1, *middle*). The case considered here is one in which the charged and uncharged lipids mix ideally in the absence of bound ligand. Formally, the binding can be considered as a two-step process (Cutsforth et al., 1989; Mosior and McLaughlin, 1992a). The ligand first absorbs to the homogeneously charged surface with statistical arrangement of lipids. Then successive rearrangements of the lipids take place in the membrane plane, resulting in the free energy change $\Delta F_{LD}(i)$. The first step is described by Eqs. 6–8. The second step will be described by the mass action law and the change in mixing entropy resulting from the accompanying lipid redistribution, which is given by the appropriate combinatorial term.

For the competitive lipid binding to a protein P at the surface of a membrane consisting of two lipid species A and B,

$$A + P \cdot B \Leftrightarrow P \cdot A + B$$

the relative binding constant is given by:

$$K_r = \frac{[P \cdot A][B]}{[A][P \cdot B]} = \frac{f_A^b (1 - f_A - \theta + f_A^b)}{(f_A - f_A^b)(\theta - f_A^b)} \quad (9)$$

where f_A is the fraction of total lipid that is of type A and θ is the fraction of the total lipid to which protein is bound. The relative binding constant K_r (>1) describes the preference of the protein for the charged lipid species A over the uncharged lipid species B. The fraction, f_A^b, of lipids that are of type A and to which protein is bound is given from Eq. 9 by:

$$f_A^b = \frac{1}{2}\left[\frac{1}{K_r - 1} + f_A + \theta - \sqrt{\left(\frac{1}{K_r - 1} + f_A + \theta\right)^2 - \frac{4K_r f_A \theta}{K_r - 1}}\right] \quad (10)$$

where the negative sign of the square root ensures that $f_A^b = 0$ for $\theta = 0$ or $f_A = 0$ ($K_r > 1$).

The change in free energy arising from the lipid redistribution on binding of the protein can be obtained from the partition function for the system of $N = n\alpha$ lipids of which $N_A = f_A N$ are of type A:

$$Q = \sum_{N_{\theta,A}} \Omega_{N_{\theta,A}}(N_\theta, N_A) K_r^{N_{\theta,A}} \quad (11)$$

where $N_\theta = \theta N$ is the total number of lipids to which protein is bound and $N_{\theta,A} = f_A^b N$ is the number of these lipids that are of type A. (It is assumed that all lipid sites associated with the protein are equivalent and noninteracting.) The combinatorial term $\Omega_{N_{\theta,A}}$ is given by the number of ways for distributing $N_{\theta,A}$ lipids of type A among the N_θ protein association sites and $N_A - N_{\theta,A}$ lipids of type A among the $N - N_\theta$ sites in the remaining lipid matrix:

$$\Omega_{N_{\theta,A}}(N_\theta, N_A) = \frac{N_\theta!}{(N_\theta - N_{\theta,A})!\, N_{\theta,A}!} \frac{(N - N_\theta)!}{[N - N_\theta - (N_A - N_{\theta,A})]!\, (N_A - N_{\theta,A})!} \quad (12)$$

As shown in the Appendix, this statistical thermodynamic formulation (Eqs. 11 and 12) is consistent with the mass action formulation given by Eq. 9 above. The change in free energy upon lipid redistribution is given by the difference from the reference state in which the lipids are homogeneously distributed, i.e., from Eq. 11:

$$\Delta F_{\mathrm{LD}}(N_\theta, N_\mathrm{A}) = -kT \ln\left[\frac{\Omega_{\mathrm{N}_{\theta,\mathrm{A}}}(N_\theta, N_\mathrm{A})}{\Omega_{\mathrm{N}^{\mathrm{o}}_{\theta,\mathrm{A}}}(N_\theta, N_\mathrm{A})} K_\mathrm{r}^{\mathrm{N}_{\theta,\mathrm{A}}-\mathrm{N}^{\mathrm{o}}_{\theta,\mathrm{A}}}\right] \tag{13}$$

where $N^{\mathrm{o}}_{\theta,\mathrm{A}} = f_\mathrm{A}\theta N$ is the number of lipids of type A to which protein is bound in the absence of lipid redistribution. In Eq. 13, the partition function is represented by its largest term. For large N, the factorials in the combinatorial terms can be approximated by Stirling's formula (i.e., $\ln N! = N \ln N - N$). The derivative of the resulting expression for the change in free energy with respect to the number i of proteins bound is then given by:

$$-\frac{d}{di}\left(\frac{\Delta F_{\mathrm{LD}}(i)}{kT}\right) = \alpha \ln\left(\frac{\theta}{1-\theta}\cdot\frac{f_\mathrm{A} - f^{\mathrm{b}}_\mathrm{A}}{f^{\mathrm{b}}_\mathrm{A}}\right) + \alpha(1-f_\mathrm{A})\ln K_\mathrm{r} \tag{14}$$

where the identity $d\theta/di = 1/n$ has been used and $f^{\mathrm{b}}_\mathrm{A}$ is given by Eq. 10.

The isotherm for binding to a mixed lipid membrane in which the charged and uncharged lipids initially are homogeneously distributed is then given in the Van der Waals approximation by (cf. Eqs. 1 and 6):

$$[L] = \frac{1}{K(0,f_\mathrm{A})}\cdot\left(1-\frac{\theta\cdot Z}{f_\mathrm{A}\cdot\alpha}\right)^{-2Z} \cdot\frac{\theta}{1-\theta}\cdot\exp\left(\frac{\theta}{1-\theta} - 2a\theta\right)\cdot\exp\left(\frac{d}{di}\left(\frac{\Delta F_{\mathrm{LD}}}{kT}\right)\right) \tag{15}$$

and the corresponding result using the SPT is given by (cf. Eqs. 1 and 7):

$$[L] = \frac{1}{K(0,f_\mathrm{A})}\cdot\left(1-\frac{\theta\cdot Z}{f_\mathrm{A}\cdot\alpha}\right)^{-2Z} \cdot\frac{\theta}{1-\theta}\cdot\exp\left(\frac{3\theta}{1-\theta} + \left(\frac{\theta}{1-\theta}\right)^2\right)\cdot\exp\left(\frac{d}{di}\left(\frac{\Delta F_{\mathrm{LD}}}{kT}\right)\right) \tag{16}$$

where f_A is the fraction of charged lipid and the final term that represents the lipid redistribution upon protein binding is given by Eq. 14. For the initial stages of ligand binding (i.e., $\theta \to 0$), the limiting case of Eq. 14 is given by:

$$-\frac{d}{di}\left(\frac{\Delta F_{\mathrm{LD}}(\theta\to 0)}{kT}\right) = \alpha[\ln(1 - f_\mathrm{A} + f_\mathrm{A}\cdot K_\mathrm{r}) - f_\mathrm{A}\cdot\ln K_\mathrm{r}] \tag{17}$$

The corresponding expression for the binding isotherm at low degrees of surface coverage is given by (cf. Eqs. 15 and 16 with $\theta \ll 1$):

$$[L] = \frac{\theta}{K(0,f_\mathrm{A})}\cdot\frac{K_\mathrm{r}^{f_\mathrm{A}\cdot\alpha}}{(1-f_\mathrm{A}+f_\mathrm{A}\cdot K_\mathrm{r})^\alpha} \equiv \frac{\theta}{K(0,f_\mathrm{A})\cdot K_{\mathrm{LD}}(0,f_\mathrm{A},K_\mathrm{r})} \tag{18}$$

where the second identity defines an "intrinsic" equilibrium constant, $K_{\mathrm{LD}}(0, f_\mathrm{A}, K_\mathrm{r})$, for the lipid redistribution at low degrees of protein binding. For the two limiting cases of $f_\mathrm{A} = 1$ and $f_\mathrm{A} = 0$, $K_{\mathrm{LD}}(0, f_\mathrm{A}, K_\mathrm{r})$ is equal to unity (see Fig. 2). At intermediate values of f_A, $K_{\mathrm{LD}}(0, f_\mathrm{A}, K_\mathrm{r})$ is greater than unity and reaches a maximum at $f_\mathrm{A}^{\max} = 1/\ln K_\mathrm{r} - 1/(K_\mathrm{r} - 1)$ because lipid rearrangement is energetically favorable whenever $K_\mathrm{r} > 1$.

The limiting form of the binding isotherm for low degrees of surface occupancy (Eq. 18) has considerable practical utility because it allows determination of the basic parameters of the system from the initial slopes of the binding isotherms. For a membrane consisting wholly of charged lipids, the intrinsic binding constant $K(0, f_\mathrm{A})$, i.e., $K(0)$, can be determined for $f_\mathrm{A} = 1$. The value of $K(0, f_\mathrm{A})$ for mixed lipid membranes, in which $f_\mathrm{A} < 1$, can then be predicted from Eq. 8. Measurements on the mixed lipid membranes

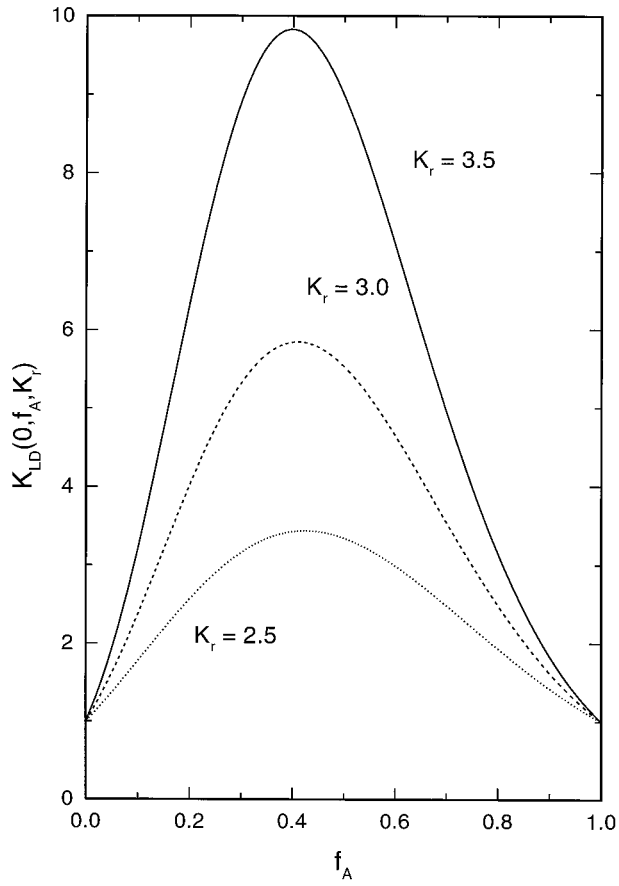

FIGURE 2 Dependence of the equilibrium constant for lipid redistribution, $K_{\mathrm{LD}}(0, f_\mathrm{A}, K_\mathrm{r})$, on the fraction, f_A, of charged lipid in mixed membranes. The dependence is calculated from Eq. 18 for relative binding constants $K_\mathrm{r} = 3.5$ (*full line*), 3.0 (*dashed line*) and 2.5 (*dotted line*), and $\alpha = 11.9$.

then allow $K_{LD}(0, f_A, K_r)$, and hence the relative lipid binding constant K_r, to be determined from the identity given in Eq. 18. To obtain the latter (i.e., K_r), it is necessary to know the lipid/protein binding stoichiometry, α, which is obtained from the saturation behavior at high protein concentrations for the single lipid system.

RESULTS

Binding isotherms have been measured for the association of cytochrome *c* with DOPG:DOPC mixed lipid membranes containing different mole fractions of DOPG. This has been done in order to determine the effects of protein binding on the lateral distribution of the charged lipid component in the membrane. Experiments have been performed for membranes of different DOPG contents and at two different ionic strengths in order to check the predictions of the model used to determine the degree of lipid redistribution on binding, which yields the relative affinities of the lipids for the protein. First, however, it is necessary to determine the ionic strength-dependence of the initial binding in order to determine the effective charge on the protein that characterizes the long-range electrostatic interactions according to double-layer theory (cf. Heimburg and Marsh, 1995). The effective protein charge parameterizes the idealized electrostatic double-layer theory in terms of the experimental system under study. In particular, this makes allowance for the finite size of the protein ligand relative to the Debye length that characterizes the ionic screening, and for the discrete nature of the protein charge distribution on the membrane surface.

Initial binding

The binding constants for the initial stages of binding at low ligand concentration were obtained over a range of ionic strengths from $[Na^+] = 40$ mM upwards, in which region the isotherms are monophasic (cf. Heimburg and Marsh, 1995). These values are defined experimentally as $[\text{cyt.}c_{\text{bound}}]/[\text{cyt.}c_{\text{free}}] \cdot [\text{lipid}]$, which differ only by a fixed factor α (corresponding to the size of the protein binding site) from those defined in the theoretical section. The data are given in Fig. 3 as a function of ionic strength for membranes of DOPG alone and for mixed membranes composed of DOPG:DOPC (60:40 mol/mol). A linear dependence is obtained in the double logarithmic plot, as predicted from Eqs. 8 and 18, for both membrane systems. Numerically, the dependence obtained for membranes composed of DOPG alone is given by (cf. Eq. 8):

$$\ln(K(0)) = \ln 0.315\alpha - 4.11 \ln c \qquad (19)$$

where the univalent cation concentration, c, is referred to a standard state $c_o = 1$ M, yielding the ionic strength. For the mixed DOPG:DOPC (60:40 mol/mol) membranes, the corresponding ionic strength dependence is given by (cf. Eq. 18):

$$\ln(K) = \ln 0.315\alpha - 4.11 \ln c + 2Z \ln f_A + \alpha[\ln(1 - f_A + f_A \cdot K_r) - f_A \cdot \ln K_r] \qquad (20)$$

where the additional terms correspond to reduction in anionic lipid content and the lateral redistribution of the lipids on protein binding, respectively. The linearity in the mixed membrane case and the identity of slope with that for DOPG membranes alone imply that the local relative association constant K_r does not depend appreciably on the ionic strength.

The gradients of the ionic strength dependence, $-Z - 0.0315Z^2$, in Eqs. 19 and 20 yield an effective protein charge of $Z = 3.7$ that governs the electrostatic enrichment of the protein concentration at the membrane surface. This effective value of Z is the same for the single- and mixed-lipid membrane systems. The absolute values of the binding constants are smaller for the mixed-lipid system than for

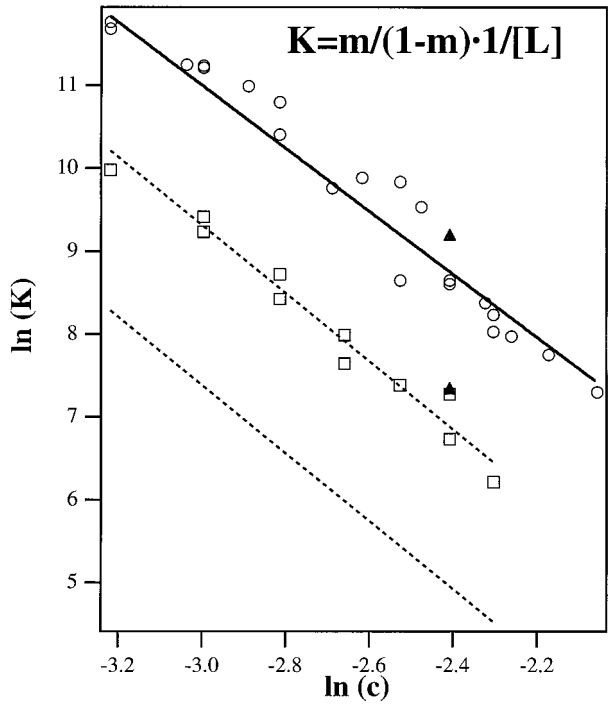

FIGURE 3 Ionic strength (c) dependence of the binding constants K/α [l/mol lipid] obtained from the initial slopes of the binding isotherms for cytochrome *c* binding to DOPG membrane dispersions (○) and to DOPG:DOPC (60:40 mol/mol) mixed membrane dispersions (□). In each case the total lipid concentration is 210 μM. The straight lines are linear regressions for the double logarithmic plots according to Eqs. 8 and 18, yielding an effective value of $Z = 3.7$ in both cases. From comparison with the data for DOPG alone, a relative binding constant of $K_r = 3.3$ (for $\alpha = 13$) or $K_r = 4.8$ (for $\alpha = 7.8$) is deduced for the DOPG:DOPC mixture by using Eq. 18. These two values of α are deduced from the VdW and SPT isotherms, respectively, in the saturation region for DOPG alone (see Fig. 4). The lower dotted line represents the values predicted for the DOPG:DOPC mixture from Eq. 8, assuming a homogeneous distribution without lipid rearrangement on protein binding (i.e., $K_r = 1$). The filled triangles represent the values of the binding constant used in Fig. 4 *b*.

membranes composed entirely of DOPG, as is expected. However, the binding constants for DOPG:DOPC (60:40 mol/mol) mixed membranes are considerably greater than would be predicted for a homogeneous lipid mixture. The latter, obtained from Eq. 20 with $K_r = 1$, are shown by the lower dashed line in Fig. 3. Taking values of the lipid stoichiometry, α, deduced from the complete binding isotherm for membranes of DOPG alone (see later) allows determination of the surface association constant for DOPG relative to DOPC: $K_r = 3.3$ for $\alpha = 13$ (VdW) and $K_r = 4.6$ for $\alpha = 7.8$ (SPT). These values for the relative affinities are deduced from the difference between the two dashed lines in Fig. 3 by using Eq. 18.

The value obtained for the effective protein charge of $Z = 3.7$ for interaction with both DOPG and mixed DOPG/DOPC membranes deserves some comment. This is less than the formal net change of +9 on the protein (Heimburg and Marsh, 1995). The positively charged residues on cytochrome *c* that can, in principle, interact with negatively charged lipid headgroups are distributed in approximately equal amounts on opposite faces of the protein (Dickerson et al., 1971). The reduced effective change for interaction with negatively charged surfaces that is parameterized by electrostatic double-layer theory therefore arises, at least in part, from the finite size of cytochrome *c* relative to the Debye length of the double layer. As already mentioned, the nonuniform surface distribution of the protein charge on the membrane also contributes to the reduction in effective charge of the protein. Using the complete expressions from electrostatic double-layer theory, rather than the high potential limit introduced in the Theory section, still yields a very similar value of the effective protein charge, $Z = 3.6$, from the data in Fig. 3. The conjugate value of the intrinsic binding constant (K_0) is correspondingly increased by a factor of ~3, but this has the compensating effect of yielding predicted binding isotherms that are practically identical to those calculated using the high potential limit.

It will be noted that the results given in Fig. 3 are free of uncertainties regarding the accessibility of lipid to protein because they are obtained only from the initial stages of protein binding.

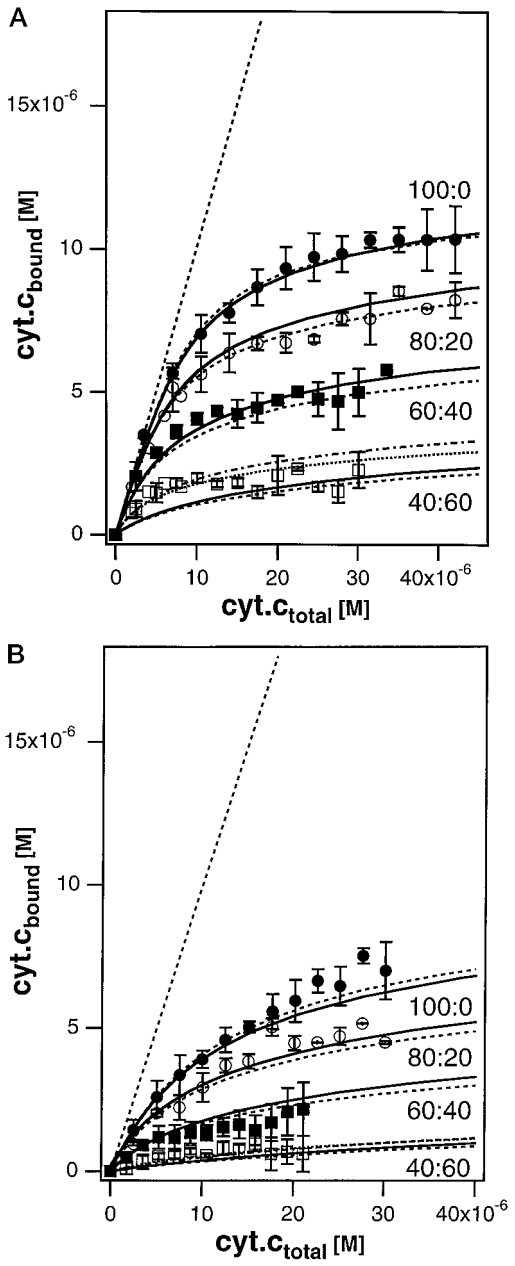

FIGURE 4 Cytochrome *c*-binding isotherms for mixed lipid membranes of the DOPG:DOPC (mol/mol) compositions shown and at ionic strengths of $[Na^+] = 45$ mM (*a*) and $[Na^+] = 90$ mM (*b*). The solid lines are SPT isotherms calculated from Eq. 16, together with Eqs. 8, 10, and 14, and the dashed lines are the corresponding VdW isotherms from Eq. 15 with $a = 0$. In each case $Z = 3.7$, and the lipid/peptide stoichiometry, α, has a value adjusted to fit the isotherms for DOPG alone (100:0) at 45 mM ionic strength in *a*. The latter yields $\alpha = 7.8$, $K_r = 4.8$ for SPT and $\alpha = 13$, $K_r = 3.3$ for VdW (see also Fig. 3). In *a*, the binding constants are obtained from the linear regressions in Fig. 3, and in *b*, the binding constants are given by the filled triangles in Fig. 3. The dashed-and-dotted and dotted lines are corresponding isotherms for DOPG:DOPC 40:60 mol/mol calculated with $K_r = 6.5$ (SPT) and $K_r = 4.5$ (VdW), respectively.

Binding curves

The complete experimental binding isotherms for association of cytochrome *c* with DOPG:DOPC mixed membranes at mole ratios from 100:0 to 40:60 are given in Fig. 4 for two ionic strengths corresponding to $[Na^+] = 45$ mM and $[Na^+] = 90$ mM. First, the binding isotherm for DOPG alone at $[Na^+] = 45$ mM was fitted to Eqs. 6 and 7 by using values of $K(0)$ and Z obtained from Fig. 3. This yields values for the lipid stoichiometry of $\alpha = 13$ from the VdW isotherm (Eq. 6) and $\alpha = 7.8$ from the SPT isotherm (Eq. 7). Both models yield equally good fits to the experimental binding isotherm for DOPG alone, but differ in the lipid stoichiometry deduced because of the difference in treatment of the excluded area terms (cf. Chatelier and Minton, 1996). For the VdW isotherm it was assumed that $a = 0$ for the protein-protein interaction term, as found previously for native cytochrome *c* (Heimburg and Marsh, 1995).

Taking these values of α from the DOPG isotherm, it is then possible to obtain the value of K_r, which governs the lipid redistribution on protein binding, as was described in the previous section (see Fig. 3 legend). Using this value together with the other parameters, $K(0)$ and Z, derived from Fig. 3, it is then possible to predict the isotherms for binding cytochrome *c* to the various lipid mixtures at different ionic strengths. This is done by using the full isotherms given by Eq. 15 or 16 for the two models, together with Eqs. 8, 10, and 14. The results of these predictions for an ionic strength specified by $[Na^+] = 45$ mM are compared with the experimental binding isotherms in Fig. 4 *a*. The predictions agree quite well with the measured values for both models, VdW and SPT, within the experimental accuracy without making any adjustments in the parameters. Only at lower contents of charged lipid and low degrees of surface coverage are some discrepancies seen. The latter can be allowed for by conservative adjustment of the fixed values of K_r that were used in predicting the isotherms. Simply increasing K_r by less than 50% produces a much better agreement with the experimental isotherms for mixtures with high DOPC content. The predictions with self-consistent values of $K_r = 6.5$ (SPT isotherm) and $K_r = 4.5$ (VdW isotherm) are given in Fig. 4 *a* for the DOPG:DOPC 40:60 mol/mol mixture that shows the largest discrepancies. Correspondingly smaller adjustments in K_r for the DOPG:DOPC 60:40 mol/mol mixture would also improve the agreement. Possibly there is a limited but progressive increase in K_r, corresponding to an increased intrinsic strength of interaction with the PG component, as the PC content in the mixtures increases. It should also be noted, however, that the experimentally determined degree of binding may be underestimated at high cytochrome *c* concentrations for lipid mixtures with high DOPC content. This is because of potential problems with accessibility that might not have been fully allowed for by the different sample preparation protocols used (see Materials and Methods).

Corresponding isotherms for a higher ionic strength specified by $[Na^+] = 90$ mM are shown in Fig. 4 *b*. In this case, the degree of protein binding is considerably lower than for $[Na^+] = 45$ mM. A somewhat better agreement with the measured data is obtained by increasing the value of the intrinsic binding constant, K_0, in the isotherms predicted for $[Na^+] = 90$ mM. The binding isotherms given in Fig. 4 *b* were calculated by increasing the constant argument of the logarithm in Eqs. 19 and 20 from 0.315 to 0.500. The corresponding values of the binding constant are given by the filled triangles in Fig. 3. It is seen that they lie practically within the range of experimental uncertainty for these values.

The experimental binding isotherms for DOPG:DOPC (60:40 mol/mol) mixed lipid membranes are compared in Fig. 5 with predictions for the two extreme cases of the lateral lipid distribution that are depicted by the top and bottom parts of Fig. 1. In both cases, the value of $Z = 3.7$ obtained from Fig. 3 and the values of α obtained from the isotherm for DOPG alone in Fig. 4 *a* are used in calculating the theoretical isotherms. It is again assumed that $a = 0$ for the VdW approximation. For a homogeneous lipid mixture, the binding isotherms are calculated from Eqs. 6 and 7,

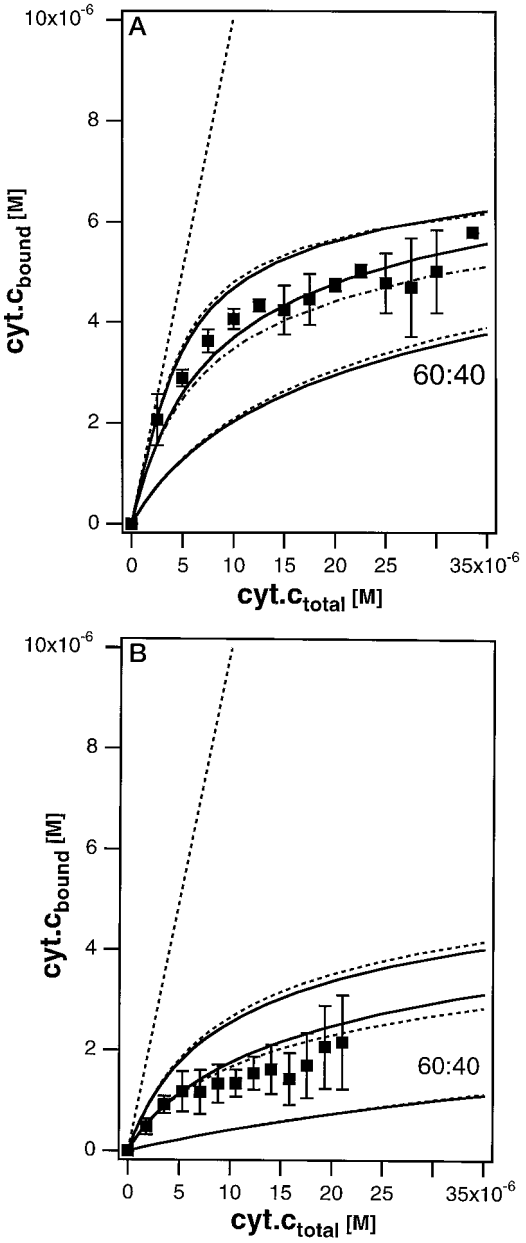

FIGURE 5 Comparison of the experimental isotherms for binding of cytochrome *c* to DOPG:DOPC (60:40 mol/mol) membranes at ionic strengths of $[Na^+] = 45$ mM (*a*) and $[Na^+] = 90$ mM (*b*), with predictions from the three models for the lipid distribution. Parameters used are given in the legends to Figs. 3 and 4. Solid lines are for the SPT model and dashed lines are for the VdW approximation. The two lowest curves are isotherms for binding to a homogeneous mixture of charged and zwitterionic lipids, given by Eq. 6 with $a = 0$ (VdW), or by Eq. 7 (SPT) and Eq. 8. The two uppermost curves are isotherms for binding to membranes with complete macroscopic demixing of the charged and zwitterionic lipids. These latter isotherms are given by the same equations as for homogenous mixing but with θ replaced by θ/f_A, and elsewhere $f_A = 1$, as described in the text. The middle curves are the isotherms for redistribution of lipids on protein binding according to the law of mass action, as given by Eq. 15 with $a = 0$ (VdW), or Eq. 16 (SPT), together with Eqs. 8, 10, and 14.

corresponding to the VdW and SPT isotherms, respectively. These predictions are given by the lowermost curves in Fig. 5. It is seen that, over the entire range of cytochrome *c* concentrations, the degree of binding measured is much greater than that expected if the lipids remain mixed homogeneously. This is in agreement with the conclusions already reached for the initial regime of low protein concentrations (Fig. 3). For a complete macroscopic demixing of the negatively charged and zwitterionic lipids, the binding isotherms are given by Eqs. 6 and 7 in which θ is replaced by θ/f_A and $f_A = 1$, at each explicit occurrence in these equations (see Theory section). These predictions are given by the uppermost curves in Fig. 5. The measured degree of binding is seen to be less than that predicted for this other extreme case at both ionic strengths. The experimental binding isotherms lie between those predicted by the two extreme models and correspond to a partial demixing of the lipids on protein binding that can be described satisfactorily by the law of mass action.

Limiting binding

The degree of surface coverage obtained in the presence of an excess of cytochrome *c* (roughly equal to the total number of protein binding sites) is given in Fig. 6 as a function of the mole fraction of charged lipid, f_{DOPG}, for membranes at two different ionic strengths. Under these conditions, the preponderance of the protein is free in solution. A sigmoidal-like dependence of the cytochrome *c* binding on the DOPG content of the membranes is obtained. Qualitatively similar results on this apparent cooperativity of lipid binding have been obtained for the association of pentalysine and related peptides with PG:PC mixed membranes (Mosior and McLaughlin, 1922a,b). Predictions of the degree of binding at stoichiometric concentrations of cytochrome *c* obtained from the three different models for the lipid distribution are also given in Fig. 6. The measured dependence on negatively charged lipid content of the membrane clearly is not compatible with a complete macroscopic demixing of the lipid components. For the latter to be the case, a strictly linear dependence on f_{DOPG} is required. Retention of a homogeneous mixture of the lipid components yields a sigmoidal-like dependence on f_{DOPG} but underestimates the degree of binding for the parameters established from Figs. 3 and 4 *a*. The dependence of cytochrome *c* binding on the mole fraction of the DOPG component can be described almost quantitatively, however, by the model that assumes a lateral redistribution of DOPG and DOPC according to their relative local affinities for cytochrome *c*. Electrostatic gathering at the membrane surface coupled to lateral redistribution of the lipids under the influence of the bound protein are sufficient to account for the apparent cooperativity, as was pointed out previously (Mosior and McLaughlin, 1992a).

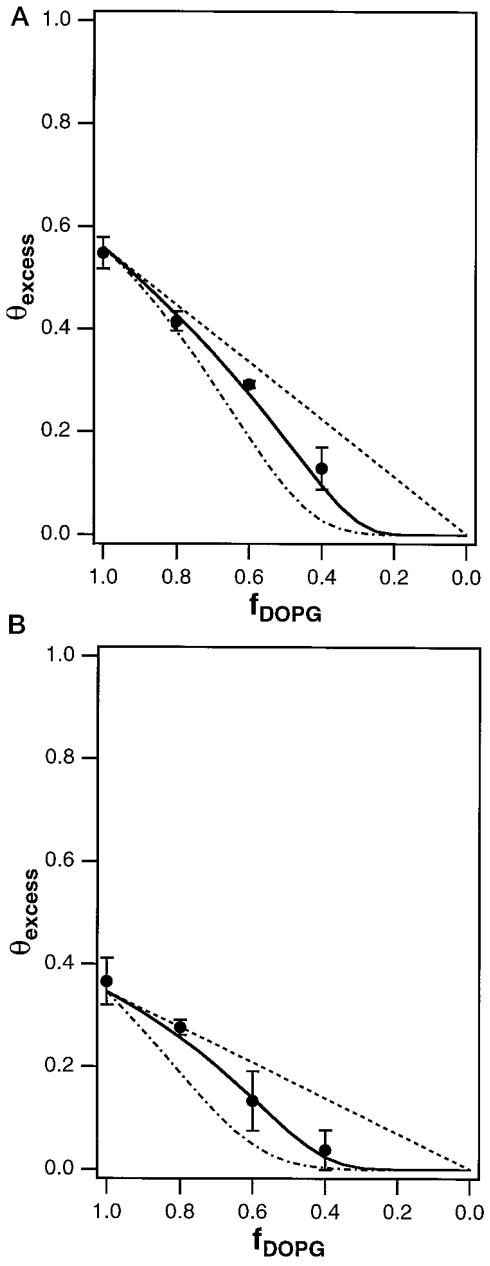

FIGURE 6 Fractional surface binding, θ_{excess}, of cytochrome *c* to DOPG:DOPC mixed membranes (210 μM lipid), as a function of mole fraction, f_{DOPG}, of DOPG in the presence of an excess (20 μM) of cytochrome *c* added, at ionic strengths of a) $[Na^+] = 45$ mM and b) $[Na^+] = 90$ mM. Data are compared with predictions from the three models for the lipid distribution, using the parameters given in the legends to Figs. 3 and 4. The middle curve is calculated from Eq. 15 with $a = 0$ (together with Eqs. 8, 10, and 14) and corresponds to lipid redistribution on protein binding. The upper and lower curves correspond to macroscopic demixing and homogeneous mixing of the lipids, respectively, and are obtained from Eq. 6 with modifications described in the text for the former case. Predictions from the SPT model are not shown but are very close to those given for the VdW model approximation.

DISCUSSION

The analysis of the cytochrome *c* binding isotherms for DOPG:DOPC mixed membranes indicates that a redistribu-

tion of the lipids takes place within the plane of the membrane on binding the protein. Phosphatidylglycerols and phosphatidylcholines with identical acyl chains are known to mix very well in hydrated membranes (Findlay and Barton, 1978; Garidel et al., 1997). Nevertheless, the binding of cytochrome *c* to DOPG:DOPC membranes is considerably greater than that predicted for a homogeneous lipid mixture. Complete demixing of the two lipid components is not achieved in the presence of an excess of cytochrome *c*, however, because the extent of binding is less than that predicted for this limiting case. Instead, the augmentation in binding, above that for homogeneously mixed lipids, can be described by a lateral redistribution of the lipids according to their relative affinities for the surface-bound protein.

The extent of lipid redistribution on protein binding is a significant membrane parameter that can be determined from the foregoing analysis. In Fig. 7, the fractional lipid composition of the membrane regions to which the protein is bound is compared with the total lipid composition of the mixed membrane. The fraction of the lipid associated with the protein that bears a charge is given by f_A^b/θ, where θ is the fraction of the total lipids to which protein is bound and f_A^b is the corresponding quantity for the charged lipids. This quantity is obtained from Eq. 10 in the limit of low protein binding, i.e., for a situation where each protein binds independently to the membrane without appreciably affecting the binding of subsequent proteins. The composition of the protein-associated regions of the membrane is given for various values of the relative lipid association constant, K_r, which span those determined here for cytochrome *c* and DOPG:DOPC membranes. It is clearly seen from Fig. 7 that the enrichment in negatively charged lipid content of the protein regions is considerable, and corresponds to the reduction in dimensionality for association within the membrane surface, in comparison with that in bulk solution (cf. Mosior and McLaughlin, 1992a; Brotherus et al., 1981). For instance, for a 60:40 mol/mol DOPG:DOPC mixed membrane the lipid associated with cytochrome *c* consists of 80–90% DOPG, and for a 40:60 mol/mol DOPG:DOPC mixture is still composed of 70% or more DOPG. At high degrees of protein binding this would cause a most appreciable depletion in negatively charged lipid of the protein-free membrane domains.

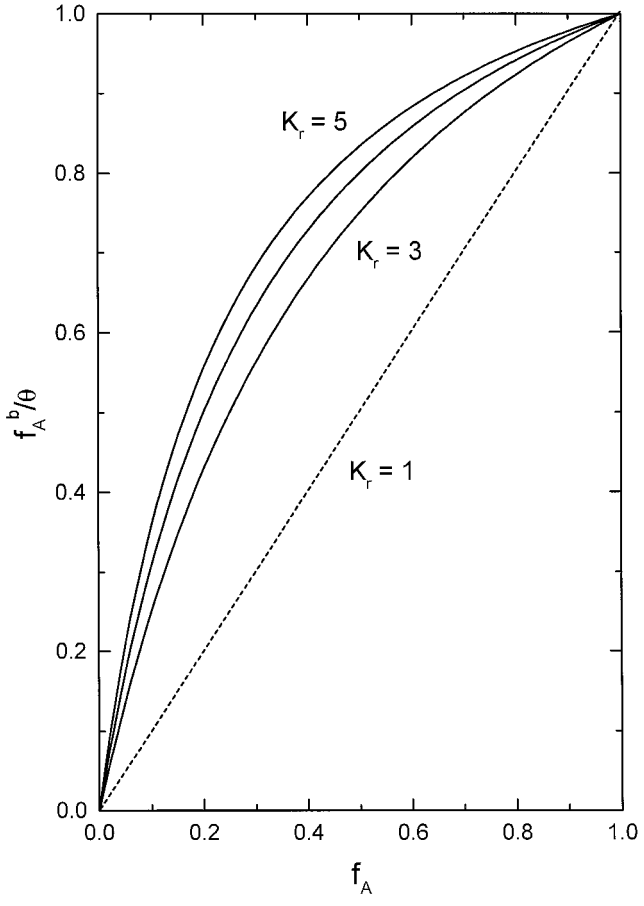

FIGURE 7 Dependence of the fraction of charged protein-associated lipid, f_A^b/θ, on the mole fraction, f_A, of charged lipid in mixed membranes containing a zwitterionic lipid component. The dependence is calculated from Eq. 10, for low levels of surface occupancy (i.e., $\theta \rightarrow 0$) and values of the relative association constant of $K_r = 3.0$, 4.0, and 5.0 (*solid lines*, lower to upper). The dashed line shows the dependence for homogeneous mixing of the two lipids.

Much emphasis in this work has been put on the inhomogeneous surface lipid distribution that results from binding of peripheral proteins to membranes. This has been derived in terms of the relative affinities of the different lipid components for the surface-bound protein and the distributional free energy arising from the combinatorial statistics of the various lipid arrangements. A somewhat similar approach to the membrane binding of peripheral proteins has been introduced by Mosior and McLaughlin (1992a) and by Cutsforth et al. (1989). The relative lipid affinities were described in the former case in terms of a three-dimensional intrinsic binding constant that was modulated by an effective surface concentration of the lipids, and in the latter case by a surface binding constant defined in terms of the mole fraction of the membrane lipid components. Here, on the other hand, we have used a relative lipid binding constant, which has the advantage that it may be compared directly with corresponding data obtained for the selectivity of lipid interactions with integral membrane proteins (Marsh, 1985) and leads in a natural way to a description of the lipid distributional statistics. Interestingly, it is concluded from the data presented in Fig. 3 that the relative surface association constant, K_r, does not depend appreciably on ionic strength. No deviations are observed from the ionic strength-dependence predicted for the electrostatic enhancement in surface protein concentration that could be attributed to an ionic strength-dependence of K_r (cf. Eqs. 8 and 18). This arises from the local surface nature of the lipid exchange equilibrium. The bound protein presumably is closely associated with the lipid headgroups such that intervening ions which might screen their mutual interaction are excluded from this region. Evidence has previously been advanced that partial dehydration of the

surface takes place on binding cytochrome *c* to negatively charged lipid membranes (Sankaram et al., 1990), which is consistent with this latter conclusion.

Determination of the relative association constant, K_r, requires knowledge of the lipid stoichiometry, α, of the protein binding region. The latter has been obtained from two separate models for the full protein binding isotherms by using either a Van der Waals description or a hard-disc treatment based on SPT. These give rise to two different values of α and hence of the lipid binding constant, K_r. The VdW approach is thought to underestimate the steric repulsions between bound proteins (Chatelier and Minton, 1996), whereas SPT somewhat overestimates hard-disc pressures (Boublík, 1975). The lipid-protein stoichiometry obtained from the VdW approximation, $\alpha = 13$, is somewhat at the high end of the values expected for cytochrome *c* binding, and that from SPT, $\alpha = 7.8$, is appreciably smaller than is expected on geometric grounds. Globular cytochrome *c* can be approximated as a prolate ellipsoid of dimensions $3.0 \times 3.4 \times 3.4$ nm (Dickerson et al., 1971), which corresponds to a cross-sectional area of 8–9 nm^2 facing the lipid surface. This value must be increased by a factor of $\sim 2\sqrt{3}/\pi$, corresponding to the area occupied by closely packed circular discs. The surface area occupied by a DOPC molecule is in the range 0.70–0.82 nm^2 (Marsh, 1990). Thus, the values predicted for the lipid stoichiometry are in the range $\alpha = 11$–14 DOPG/DOPC molecules per cytochrome *c*. It therefore seems likely that the value for the relative lipid binding constant lies intermediate within the range K_r(PG:PC) = 3.3–4.8 that is determined for the VdW and SPT stoichiometries, respectively. Discrimination between the VdW and SPT models cannot be made on the basis of the binding isotherms, other than in the quantitative values derived for α, because both models describe the experimental results equally well.

It should be noted that estimation of the relative association constant K_r from the data given in Fig. 3 requires use of the electrostatic model as expressed in Eq. 8. The reliability of these values therefore rests on the extent to which experimental parameterization by means of the effective value of the protein charge, *Z*, accounts for the limitations of the simple double-layer theory with respect to finite protein size and discreteness of protein distribution on the membrane surface. The consistency of the predictions of the model with the full experimental binding curves determined under a variety of conditions (Fig. 4), when conservative adjustments are made in the relative association constant (K_r), suggests that a reasonable degree of reliability is achieved.

The intrinsic surface affinity for cytochrome *c* of phosphatidylglycerol relative to phosphatidylcholine corresponds to a free energy difference of $\Delta G(\text{PG:PC}) = -RT \ln K_r = -(3.1\text{–}4.0)$ kJ/mol at 37°C. This relatively modest free energy difference, however, gives rise to a strong preferential association of DOPG with cytochrome *c*, as seen in Fig. 7, because of the high effective surface concentrations in the membrane. Correspondingly, this preferential affinity for DOPG greatly enhances the membrane binding of cytochrome *c*, as seen already from Fig. 5. Formally, this enhanced binding is described by the composite equilibrium constant $K_{LD}(0, f_A, K_r)$ (see Fig. 2) that depends on both the relative binding constant K_r and the lipid stoichiometry α.

The values for the relative association constant and free energy of interaction of phosphatidylglycerol with cytochrome *c* that are determined here lie within the range of those found for the selective interaction of different spin-labeled phospholipid species with integral membrane proteins (Marsh, 1985, 1995). Although the latter mostly display a specificity for anionic lipids, an interesting feature is that phosphatidylglycerol generally does not display a selectivity of interaction with integral proteins relative to phosphatidylcholine. This difference between the two types of membrane proteins probably lies not only in a different amino acid disposition, but also in the different topography of the lipid interactions with peripheral and integral proteins, respectively. As mentioned in the Introduction, several spectroscopic studies have indicated a preferential in-plane interaction of negatively charged phospholipids with peripheral proteins. In ESR studies, these have been used to establish a hierarchy of interactions of spin-labeled lipids (at probe amounts) with a range of peripheral proteins (Sankaram et al., 1989, 1990). With certain assumptions, the latter were also used to obtain approximate values for the relative association constants: a value of K_r(PG:PC) ~2 was estimated for the interaction of phosphatidylglycerol relative to phosphatidylcholine with cytochrome *c*. The present thermodynamic determinations suggest that the assumptions made in these approximations must be modified and that the values of K_r obtained from ESR must be corrected upward. Spin-labeled lipids that experience a greater perturbation than phosphatidylglycerol on binding cytochrome *c*, e.g., phosphatidylinositol, are, however, expected to have larger values of K_r than those reported here.

This study has been devoted to pairs of lipids that mix well in the absence of protein, and therefore constitutes the most sensitive test of the model used to describe the influence of lipid redistribution on protein binding. It will be noted, however, that the treatment applies also to lipids that have an inherent tendency either to phase separation or to a more uniform ordered distribution. In these latter cases, the protein binding will tend more to be determined by the lipid mixing properties than by the relative selectivity of interaction with the protein.

This work has been supported in part by the Deutsche Forschungsgemeinschaft (Ma756/8-1).

APPENDIX

Statistical thermodynamic derivation of the fraction of protein-associated lipid

Corresponding to the partition function given by Eq. 11, the free energy of the configurations with $N_{\theta,A}$ lipids of type A that are bound to the protein

is given by:

$$F_{N_{\theta,A}} = -kT \ln[\Omega_{N_{\theta,A}}(N_\theta, N_A)K_r^{N_{\theta,A}}] \quad (A1)$$

where the combinatorial term, $\Omega_{N_{\theta,A}}(N_\theta, N_A)$, is given by Eq. 12. The equilibrium condition for minimum free energy, i.e., $\partial F_{N_{\theta,A}}/\partial N_{\theta,A} = 0$, is therefore:

$$\ln K_r = -\frac{\partial \ln \Omega_{N_{\theta,A}}(N_\theta, N_A)}{\partial N_{\theta,A}} \quad (A2)$$

where the derivative of the combinatorial term can be evaluated by using Stirling's formula [i.e., $(1/N!)(dN!/dN) = \ln N$]. The resulting expression for the relative binding constant is:

$$K_r = \frac{N_{\theta,A}(N - N_\theta - N_A + N_{\theta,A})}{(N_\theta - N_{\theta,A})(N_A - N_{\theta,A})} \quad (A3)$$

which, expressed in terms of the fractional lipid populations, is identical to Eq. 9. The partition function used for determining the free energy of lipid redistribution is therefore equivalent to the law of mass action and the fraction of protein-associated lipid ($f_A^b = N_{\theta,A}/N$) is given by Eq. 10.

REFERENCES

Ben-Tal, N., B. Honig, R. M. Peitzsch, G. Denisov, and S. McLaughlin. 1996. Binding of small basic peptides to membranes containing acidic lipids: theoretical models and experimental results. *Biophys. J.* 71: 561–575.

Boublík, T. 1975. Two-dimensional convex particle liquid. *Mol. Phys.* 29:421–328.

Brotherus, J. K., O. H. Griffith, M. O. Brotherus, P. C. Jost, J. R. Silvius, and L. E. Hokin. 1981. Lipid-protein multiple binding equilibria in membranes. *Biochemistry.* 20:5261–5267.

Carbone, M. A., and P. M. Macdonald. 1996. Cardiotoxin II segregates phosphatidylglycerol from mixtures with phosphatidylcholine: ^{31}P and ^{2}H NMR spectroscopic evidence. *Biochemistry.* 35:3368–3378.

Chatelier, R. C., and A. P. Minton. 1996. Adsorption of globular proteins on locally planar surfaces: models for the effect of excluded surface area and aggregation of adsorbed protein in adsorption equilibria. *Biophys. J.* 71:2367–2374.

Cutsforth, G. A., R. N. Whitaker, J. Hermans, and B. R. Lentz. 1989. A new model to describe extrinsic protein binding to phospholipid membranes of varying composition: Application to human coagulation proteins. *Biochemistry.* 28:7453–7461.

Dickerson, R. E., T. Takano, D. Eisenberg, O. B. Kallai, L. Samson, A. Cooper, E. Margoliash. 1971. Ferricytochrome *c* 1. General features of the horse and bonito proteins at 2.8 Å resolution. *J. Biol. Chem.* 246: 1511–1535.

Findlay, E. J., and P. G. Barton. 1978. Phase behavior of synthetic phosphatidylglycerols and binary mixtures with phosphatidylcholines in the presence and absence of calcium ions. *Biochemistry.* 17:2400–2405.

Garidel, P., C. Johann, L. Mennicke, and A. Blume. 1997. The mixing behavior of pseudobinary phosphatidylcholine-phosphatidylglycerol mixtures as a function of pH and chain length. *Eur. Biophys. J.* 26: 447–459.

Gawrisch, K., J. A. Barry, L. L. Holte, T. Sinnwell, L. D. Bergelson, and J. A. Ferretti. 1995. Role of interactions at the lipid-water interface for domain formation. *Mol. Membr. Biol.* 12:83–88.

Haverstick, D. M., and M. Glaser. 1989. Influence of proteins on the reorganization of phospholipid bilayers into large domains. *Biophys. J.* 55:677–682.

Heimburg, T., and D. Marsh. 1995. Protein surface-distribution and protein-protein interactions in the binding of peripheral proteins to charged lipid membranes. *Biophys. J.* 68:536–546.

Helfand, E., H. L. Frisch, and J. L. Lebowitz. 1961. Theory of the two- and one-dimensional rigid sphere fluids. *J. Chem. Phys.* 14:1037–1042.

Jähnig, F. 1976. Electrostatic free energy and shift of the phase transition for charged lipid membranes. *Biophys. Chem.* 4:309–318.

Kleinschmidt, J. H., and D. Marsh. 1997. Spin-label electron spin resonance studies on the interactions of lysine peptides with phospholipid membranes. *Biophys. J.* 73:2546–2555.

Marsh, D. 1985. ESR spin label studies of lipid-protein interactions. *In* Progress in Protein-Lipid Interactions, Vol 1. A. Watts and J. J. H. H. M. de Pont, editors. Elsevier, Amsterdam. 143–172.

Marsh, D. 1990. CRC Handbook of Lipid Bilayers, CRC Press, Boca Raton, FL.

Marsh, D. 1995. Specificity of lipid-protein interactions. *In* Biomembranes, Vol. 1. A. G. Lee, editor. JAI Press, Greenwich, CT. 137–186.

Mosior, M., and S. McLaughlin. 1992a. Electrostatics and reduction of dimensionality produce apparent cooperativity when basic peptides bind to acidic lipids in membranes. *Biochim. Biophys. Acta.* 1105:185–187.

Mosior, M., and S. McLaughlin. 1992b. Binding of basic peptides to acidic lipids in membranes: effects of inserting alanine(s) between the basic residues. *Biochemistry.* 31:1767–1773.

Mosior, M., and A. C. Newton. 1995. Mechanism of interaction of protein kinase C with phorbol esters: reversibility and nature of membrane association. *J. Biol. Chem.* 270:25526–25533.

Pinheiro, T. J. T., and A. Watts. 1994. Resolution of individual lipids in mixed phospholipid membranes and specific lipid-cytochrome *c* interactions by magic-angle spinning solid-state phosphorus-31 NMR. *Biochemistry.* 33:2459–2467.

Reiss, H., H. L. Frisch, and J. L. Lebowitz. 1959. Statistical mechanics of rigid spheres. *J. Chem. Phys.* 31:369–380.

Rodgers, W., and M. Glaser. 1991. Characterization of lipid domains in erythrocyte membranes. *Proc. Natl. Acad. Sci. USA.* 88:1364–1368.

Roux, M., J. M. Neumann, M. Bloom, and P. F. Devaux. 1988. ^{2}H and ^{31}P NMR study of pentalysine interaction with headgroup deuterated phosphatidylcholine and phosphatidylserine. *Eur. Biophys. J.* 16:267–273.

Sankaram, M. B., P. J. Brophy, and D. Marsh. 1989. Selectivity of interaction of phospholipids with bovine spinal cord myelin basic protein studied by spin-label electron spin resonance. *Biochemistry.* 28: 9699–9707.

Sankaram, M. B., P. J. Brophy, W. Jordi, and D. Marsh. 1990. Fatty acid pH titration and the selectivity of interaction with extrinsic proteins in dimyristoylphosphatidylglycerol dispersions. Spin label ESR studies. *Biochim. Biophys. Acta.* 1021:63–69.

Sankaram, M. B., B. de Kruijff, and D. Marsh. 1989. Selectivity of interaction of spin-labelled lipids with peripheral proteins bound to dimyristoylphosphatidylglycerol bilayers, as determined by ESR spectroscopy. *Biochim. Biophys. Acta.* 986:315–320.

Sankaram, M. B., and D. Marsh. 1993. Protein-lipid interactions with peripheral membrane proteins. *In* New Comprehensive Biochemistry, Vol. 25. Protein-Lipid Interactions. A. Watts, editor. Elsevier, Amsterdam. 127–162.

Yang, L., and M. Glaser. 1995. Membrane domains containing phosphatidylserine and substrate can be important for the activation of protein kinase C. *Biochemistry.* 34:1500–1506.